

High focal adhesion kinase expression in invasive breast carcinomas is associated with an aggressive phenotype

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Focal adhesion kinase (FAK) is a protein tyrosine kinase expressed in invasive breast cancer that regulates antiapoptotic signaling. We have examined FAK expression by immunohistochemistry using anti-FAK 4.47 in breast tumor samples from a large population-based, case-control study of women participating in the University of North Carolina Breast Specialized Programs of Research Excellence (SPORE), Carolina Breast Cancer Study. In this population, 629 formalin-fixed, paraffin-embedded tissue sections were stained for FAK and scored as high (3+ or 4+ intensity and $\geq 90\%$ positive cells) or otherwise. High FAK expression was associated with poor prognostic indicators including high mitotic index (> 10 mitoses per 10 consecutive high-power fields), nuclear grade 3, architectural grade 3, estrogen and progesterone receptor negative, and HER-2/*neu* overexpressed using CB11 antibody. The association of high FAK expression with HER-2/*neu* overexpression lends further support that HER-2/*neu* and FAK collaborate to promote tumorigenesis. The presence of strong FAK expression in many high grade, estrogen- and progesterone-negative breast carcinomas indicates that FAK may be an attractive target for therapeutic intervention.

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Tyrosine kinases, proteins that phosphorylate substrate proteins on tyrosine residues, regulate key processes in tumorigenesis, including proliferation, angiogenesis, and cellular survival. For this reason, tyrosine kinases have the potential to predict tumor progression and serve as targets for therapeutic intervention.¹ While the expression patterns of tyrosine kinases such as HER-2/*neu* and epidermal growth factor receptor have been extensively characterized in tumors,² the functions of other tyrosine

kinases in cancer are only beginning to be understood.

Focal adhesion kinase (FAK) is a 125 kDa protein tyrosine kinase that localizes to sites of cellular adhesion in cultured cells. FAK functions in integrin-signaling pathways,^{3–6} cellular motility,⁷ and apoptosis.^{8–10} The avian FAK homologue was originally identified through its association with the v-Src oncogene protein,¹¹ suggesting a role for FAK in tumorigenesis. In our lab, human FAK was subsequently identified in high-grade sarcomas¹² and breast tumors.^{13–15} FAK is overexpressed in a variety of human tumors in comparison with adjacent normal tissue, including breast,^{13–16} colon,^{13,14,17,18} thyroid,¹⁹ ovarian,²⁰ cervix,¹⁶ head and neck,²¹ and esophagus.²²

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Early work suggested that FAK might function in the later stages of tumor progression, such as invasion and metastasis, promoting adhesion in invading cells. However, FAK expression is detected in early stage tumors, such as ductal carcinoma *in situ*,¹⁵ suggesting that FAK might have functions in cancer progression that precede invasion and metastasis. Work from this lab and others has demonstrated that FAK regulates antiapoptotic survival signaling, even in cells maintained in the absence of adhesion.^{23,24} This suggests that FAK could function in the earlier stages of tumor progression as a regulator of survival, suppressing apoptotic responses associated with uncontrolled proliferation and signaling.

In the present study, we have analyzed the expression of FAK in a large population-based study of breast tumor samples. Although we had previously shown FAK overexpression in breast tumors,^{13–15} the previous studies lacked sufficient numbers of patient samples to derive relationships between FAK expression and other important clinical markers.

Materials and methods

Study Population

The Carolina Breast Cancer Study is a population-based, case-control study whose study participants included women 20–74 years of age from 24 contiguous counties of central and eastern North Carolina.^{25,26} Using the North Carolina Central Cancer Registry, 1153 women with a first diagnosis of invasive breast cancer between 1993 and 1996 were identified.²⁷ African-American and women under the age of 50 years were over-sampled using randomized recruitment. Informed consent was obtained and 861 invasive breast cancer cases were eligible for participation in Phase 1 of Carolina Breast Cancer Study, overall response rate = 75%. Medical records or direct histopathological review of tumor tissue provided clinical data and information about tumor characteristics. The University of North Carolina School of Medicine Institutional Review Board approved all aspects of this research.

Tissue Specimens

Formalin-fixed, paraffin-embedded tumor blocks were obtained from participating hospitals' pathology departments and tumors were sectioned by the Specialized Programs of Research Excellence (SPORE) Immunohistochemistry Core facility as described previously.²⁸ All sections were stored at 4°C. Of the 861 formalin-fixed, paraffin embedded specimens received for the Carolina Breast Cancer Study, 629 were available and contained sufficient invasive cancer for FAK immunohistochemistry.

Immunohistochemistry

FAK immunohistochemistry was performed using the monoclonal antibody 4.47 (1:250; Upstate Biotechnology, Lake Placid, NY, USA) to the amino-terminal region of FAK.¹⁴ This region of FAK is preferred as an antigenic target since anti-FAK antibodies directed at the carboxy terminus of the FAK molecule do not distinguish between FAK and FAK-related nonkinase (FRNK) proteins.¹⁴ The 4.47 antibody has been previously characterized and recognizes FAK in formalin-fixed, paraffin-embedded breast tumor sections, with minimal crossreactivity.¹⁴

FAK immunohistochemical analyses were performed as described previously.¹⁸ Briefly, slides were incubated overnight with anti-FAK 4.47 monoclonal antibody (1:250; Upstate Biotechnology) after heat-induced epitope recovery and blocking with normal horse serum (Vectastatin Elite Kit; Vector Laboratories, Burlingame, CA, USA). The DAKO Autostainer (DAKO Corporation) was utilized for application and incubation of the biotinylated horse antimouse IgG (Vectastain Elite Kit, Vector Laboratories), avidin-biotin complex (Vectastain Elite Kit, Vector Laboratories), and slate gray chromagen (Vector Laboratories).

HER-2/*neu* immunohistochemistry had been previously performed in the SPORE Immunohistochemistry laboratory using clone CB11 (1:100; BioGenex, San Ramon, CA, USA).²⁹ These assays were scored previously (LG and MI) and results maintained in the SPORE Immunohistochemistry database. Immunohistochemistry was performed using an avidin-biotin immunoperoxidase system with 3',3' diaminobenzidine as chromagen. Briefly, slides were incubated overnight with clone CB11 after quenching endogenous peroxidase using a methanol-peroxide bath and blocking with normal horse serum (Vectastain Elite Kit, Vector Laboratories). During day 2, slides were incubated with biotinylated secondary antibody (Vectastain Elite Kit, Vector Laboratories), avidin-biotin complex (Vectastain Elite Kit, Vector Laboratories), and 3',3' diaminobenzidine (Sigma, St Louis, MO, USA). Cases were considered positive for HER-2/*neu* if there was unambiguous membrane staining of a weak, moderate, or strong intensity in at least 10% of the invasive cancer cells.

Immunohistochemistry Scoring for FAK

A single board-certified pathologist (CAL) scored each tissue section for FAK expression based on a scoring system that measured intensity (0, none; 1, borderline; 2, weak; 3, moderate; 4, strong), percentage positive cells (0–100), and cellular localization (nucleus, cytoplasm, membrane, or combination). In order to better define FAK expression in a large study population, we dichotomized FAK expression as high (3+ or 4+ intensity and ≥90% positive

cells) and not high (0, 1, or 2 intensity and/or <90% positive cells) (Figure 1).

Statistical Methods

Fisher's exact test was used for data categorized into two by two contingency tables. For the larger

contingency tables (ie the two by three and two by four tables of mitotic index, nuclear grade, histological grade, and stage at diagnosis by dichotomized FAK expression), we used the nonparametric Jonckheere–Terpstra method to test for ordered differences. With this test, the null hypothesis is that the distribution of the response does not differ across ordered categories. Statistical analyses were performed with SAS statistical software, Version 8.2, SAS Institute Inc., Cary, NC, USA. Logistic regression was implemented using SAS version 6.11 (SAS Institute Inc.) to estimate odds ratios for the association of high FAK expression with the following potential predictive variables: race, menopausal status, stage at diagnosis, lymph node status, nuclear grade, histologic grade, mitotic index, HER-2/*neu* expression, and estrogen receptor (ER) status. Models were adjusted for age.

Results

Formalin-fixed, paraffin-embedded tumor blocks were obtained for 629 (73%) of 861 tumors of breast cancer patients who consented to participate in Phase 1 of Carolina Breast Cancer Study. Among the 629 Carolina Breast Cancer Study breast cancer patients, 41% were African-American and 52% were premenopausal³⁰ (Table 1). The mean age was 48 years (minimum age 23 years, maximum age 74 years; Table 1). The majority of these women presented with stage 1 or 2 disease (89%)³¹ and negative lymph nodes (60%, Table 1). The majority of the breast cancers were poorly differentiated (65%) with moderate to marked nuclear pleomorphism (88%, Table 1), ER (59%) and progesterone receptor (PR; 57%) positive, and negative for HER-2/*neu* overexpression 77% (Table 2). In all, 25% of the breast tumors demonstrated high FAK expression (Table 2). In this study population, recurrence and survival data have not been compiled to date.

Tumors that expressed high levels of FAK (Table 3) were compared with other clinicopathological features. High FAK expression was highly associated with a mitotic index of >10 mitoses per 10 consecutive high-power fields ($P < 0.0001$) and nuclear grade 3 breast tumors ($P < 0.0001$). Breast tumors with high FAK expression were also more

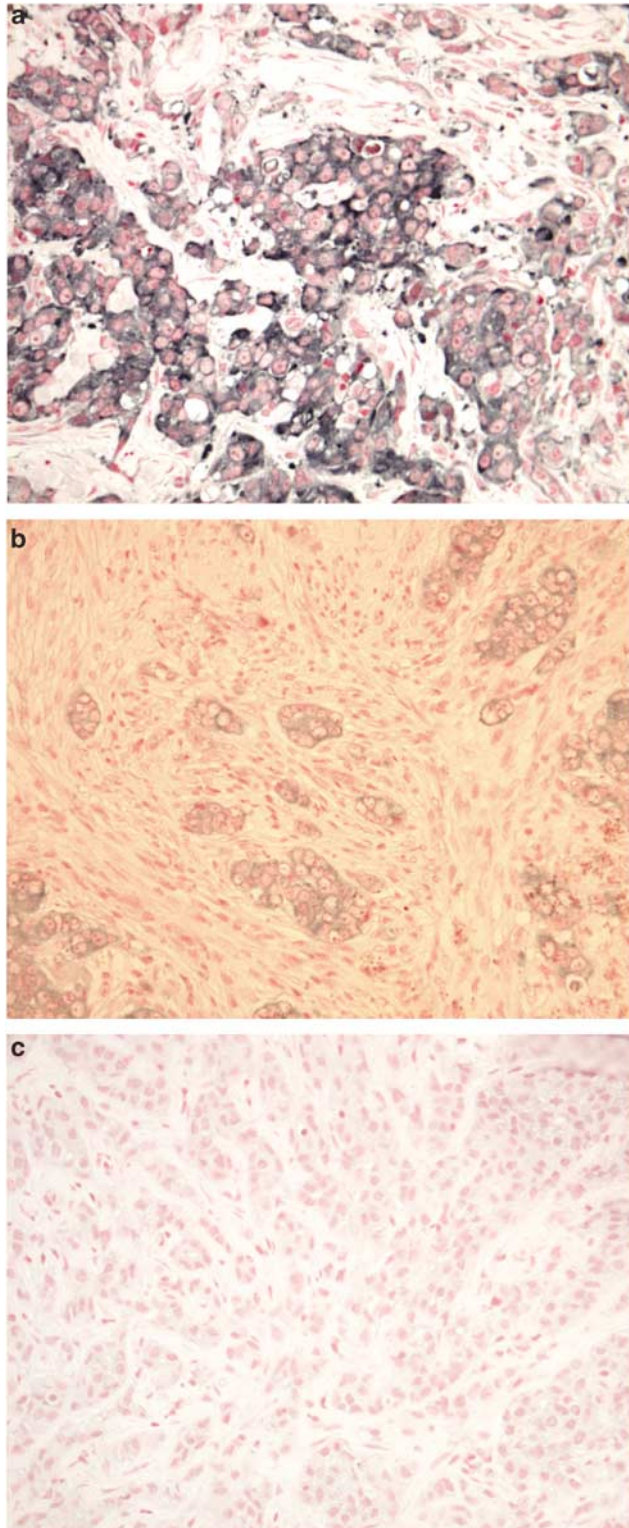


Figure 1 Immunohistochemical analysis of FAK expression in invasive breast cancer. Anti-FAK 4.47 monoclonal antibody (1:250) was used to detect FAK expression with slate gray chromagen. Tissue sections for FAK expression is based on a scoring system that measured intensity (0, none; 1, borderline; 2, weak; 3, moderate; 4, strong), percentage positive cells (0–100), and cellular localization (nucleus, cytoplasm, membrane, or combination). FAK expression was dichotomized as high (3+ or 4+ intensity and $\geq 90\%$ positive cells) or not high. (a) FAK expression in invasive breast cancer demonstrating strong cytoplasmic expression ($\times 400$ magnification). (b) Weak FAK expression demonstrated by faint cytoplasmic staining within invasive breast cancer cells ($\times 400$ magnification). (c) No FAK expression demonstrated in breast cancer cells ($\times 400$ magnification).

Table 1 Characteristics of breast cancer patients in the CBCS with FAK data

Characteristic	Number (%)
Number of cases	629
Age (mean)	48
Minimum	23
Maximum	74
Race	
African-American	261 (41%)
Non African-American	368 (59%)
Menopausal status	
Premenopausal	328 (52%)
Postmenopausal	301 (48%)
Stage at diagnosis ^a	
1. Stage I	227 (39%)
2. Stage IIA, IIB	292 (50%)
3. Stage IIIA, IIIB	50 (8%)
4. Stage IV	15 (3%)
Mitotic index	
1. ≤ 5 mitoses	264 (42%)
2. 6–10 mitoses	85 (14%)
3. > 10 mitoses	273 (44%)
Nuclear grade	
1. Slight pleomorphism	76 (12%)
2. Moderate pleomorphism	289 (46%)
3. Marked pleomorphism	261 (42%)
Histological grade	
1. (Well differentiated)	37 (6%)
2. (Moderately differentiated)	180 (29%)
3. (Poorly differentiated)	408 (65%)
Lymph node status	
Positive	239 (40%)
Negative	360 (60%)

^aAJCC Cancer Staging Handbook, 5th Edition.

Table 2 Tumor marker expression in CBCS breast cancer patients

Marker	Number (%)
FAK	
High ^a	154 (25%)
Not High ^b	475 (76%)
Estrogen receptor	
Positive	355 (59%)
Negative	251 (41%)
Progesterone receptor	
Positive	340 (57%)
Negative	261 (43%)
HER-2/neu	
Positive	145 (23%)
Negative	481 (77%)

^aFAK high = 3+ or 4+ intensity and ≥90% positive cells.

^bFAK not high = 0, 1, 2 intensity and/or <90% positive cells.

Table 3 Correlation between FAK immunohistochemical expression and clinicopathological features

Clinicopathological feature	Total	FAK high ^a	FAK not high ^b	P-value
Mitotic index				
1. ≤ 5 mitoses	264	34 (13%)	230 (87%)	
2. 6–10 mitoses	85	25 (29%)	60 (71%)	
3. > 10 mitoses	273	93 (34%)	180 (66%)	<0.0001
Nuclear grade				
1. Slight pleomorphism	76	6 (0.08%)	70 (92%)	
2. Moderate pleomorphism	289	51 (18%)	238 (82%)	
3. Marked pleomorphism	261	96 (37%)	165 (63%)	<0.0001
Histological grade				
1. Well differentiated	37	3 (0.08%)	34 (92%)	
2. Moderately differentiated	180	41 (23%)	139 (77%)	
3. Poorly differentiated	408	109 (27%)	299 (73%)	0.038
Stage at diagnosis ^c				
1. Stage I	227	45 (20%)	182 (80%)	
2. Stage IIA, IIB	292	75 (26%)	217 (74%)	
3. Stage IIIA, IIIB	50	15 (30%)	35 (70%)	
4. Stage IV	15	3 (20%)	12 (80%)	0.08
Lymph node status				
Positive	239	69 (29%)	170 (71%)	
Negative	360	79 (22%)	281 (78%)	0.066
Estrogen receptor				
Positive	355	71 (20%)	284 (80%)	
Negative	251	78 (31%)	173 (69%)	0.002
Progesterone receptor				
Positive	340	65 (19%)	275 (81%)	
Negative	261	83 (32%)	178 (68%)	0.0004
HER-2/neu				
Positive	145	49 (34%)	96 (66%)	
Negative	481	105 (22%)	376 (78%)	0.004

^aFAK high = 3+ or 4+ intensity and ≥90% positive cells.

^bFAK not high = 0, 1, 2 intensity and/or <90% positive cells.

^cAJCC Cancer Staging Handbook, 5th Edition.

likely to be architectural grade 3 tumors ($P=0.038$, Table 3).

FAK expression was also associated with several markers of poor prognosis. Breast tumors expressing high levels of FAK were associated with ER negative ($P=0.002$) and PR negative ($P=0.0004$, Table 3) phenotype. In addition, high FAK expression was associated with overexpression of the tyrosine kinase receptor HER-2/neu ($P=0.004$, Table 3). After multivariable adjustment, the only significant predictors of breast tumors with high FAK expression were HER-2/neu positivity (2.2 (1.4–3.4), $P=0.001$) and nuclear grade 3 tumors (2.3 (1.4–4.0), $P=0.002$).

In the Carolina Breast Cancer Study population, patients presenting at diagnosis with stage 3 or 4 breast cancers (65/584) (American Joint Committee on Cancer, 5th Edition, Cancer Staging Classification for Breast Cancer Stages IIIA, IIIB, or IV) were more likely to have tumors with high FAK expression ($P=0.08$). Patients presenting with positive lymph nodes were also more likely to have breast tumors with high FAK expression ($P=0.066$). Although not statistically significant, high FAK expression is common in advanced stage.

Discussion

We have examined the characteristics of a population-based, case-control study of breast cancer. High FAK expression in the Carolina Breast Cancer Study breast tumors is associated with a poor prognostic phenotype. Histologically, these tumors have a high mitotic index (<10 mitoses per high power field), grade 3 nuclear and architectural features, lack ER and PR, and overexpress HER-2/*neu*. To date, recurrence and survival data for this Carolina Breast Cancer Study population is not available in order to evaluate FAK as a prognostic marker.

Overexpression of HER-2/*neu* is usually associated with a worse prognosis in primary breast cancer and is observed in 15–30% of breast cancers.^{32–34} In the current study of Carolina Breast Cancer Study patients, 23% of breast cancers overexpressed HER-2/*neu*. The association of high FAK expression with HER-2/*neu* overexpression suggests that high FAK expression may also be a poor prognostic marker. Even though the molecular interactions between FAK and HER-2/*neu* signaling are just beginning to be understood, work by Vadlamudi *et al*³⁵ suggest that HER-2/*neu* and heregulin are involved in FAK signaling and selectively upregulate FAK tyrosine phosphorylation.

Work from this lab and others has indicated a role for FAK in survival signaling, which is the propagation of signals that are required to maintain viability and suppress apoptosis. Attenuation of FAK expression with antisense oligonucleotides leads to apoptosis in tumor cells but not in nontransformed cell lines.⁸ Subsequent work has utilized overexpression of a fragment of the FAK carboxy-terminal domain (FRNK or FAK-CD for FAK-carboxy-terminal domain) that acts as a dominant-negative protein. FRNK/FAK-CD leads to the inhibition of endogenous FAK phosphorylation³⁶ and the degradation of FAK in tumor cells.^{23,37} As a result, FRNK/FAK-CD leads to apoptotic cell death in colon³⁷ and breast cancer cells,²³ but not in normal nontumor cells.^{23,37} Furthermore, FRNK/FAK-CD inhibits apoptosis even in cells maintained in suspension, indicating a role for FAK in suppression of apoptosis even in the absence of adhesive signaling. Expression of the N-terminus of FAK in breast carcinoma cell lines

resulted in rounding, detachment and apoptosis.³⁸ Recent work has shown FAK association with receptor-interacting protein, a serine/threonine kinase that contains a death domain.³⁹ When FAK binds to receptor-interacting protein, the proapoptotic signals supplied by receptor-interacting protein are suppressed. This further supports the role of FAK as a survival signal.³⁹

In this study, high FAK expression had a borderline association with positive lymph node status and stage at diagnosis. Owing to the low number of patients presenting with stage III and IV disease we cannot confirm this association in this study. However, since high FAK was associated with other poor prognostic indicators, examining a population with higher numbers of stage III and IV disease would be worthwhile. Miyazaki *et al*²² reported an association between FAK overexpression (intense staining of >40% of carcinoma cells) and lymph node metastasis in esophageal squamous cell carcinoma. Indeed, the mechanistic framework of FAK as a survival signal would suggest a role for FAK in metastatic spread to lymph nodes and such a role has been observed in esophageal carcinoma.²²

These results also emphasize the potential of FAK as a molecular target for cancer therapeutics. Although we dichotomized FAK expression as high or not high, our cutoff for high FAK was expression in at least 90% of cells, with an intensity of 3+ or 4+. Moreover, normal breast cells express negligible levels of FAK, demonstrating a 'therapeutic window' between normal and tumor cells. Taken together, our results demonstrate the aggressive phenotype of high FAK expression and offer the potential for therapeutics designed to abrogate its function.

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